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14. ABSTRACT Our overall goal is to develop a safe and feasible model for the chemoprevention of a wide range of tobacco-related diseases. Our immediate goal, that is addressed over a 5-year study period, is to determine the effects of high tea consumption on biological markers of oxidative stress that mediate lung cancer risk. We are conducting a 6-month randomized, controlled, double-blinded chemopreventive trial in a group of COPD subjects who are being randomized to green or black tea preparations or a control intervention (matching placebo). Levels of 8-hydroxydeoxyguanosine and 8-F2-isoprostanes are used to measure DNA and lipid damage respectively. Changes in biomarkers of oxidative damage are being measured in urine, blood and exhaled breath condensate. The study protocol was approved by all parties in September 2003. Recruitment and screening of participants for eligibility criteria started in October 2003. Total recruitment was completed in December 2007. A total of 154 participants completed the study. Laboratory analyses (urine, blood and sputum) for the last cohort of participants is ongoing along with data entry. We expect to complete all the laboratory analyses by June 2008 and the data analyses and final report and manuscript preparation will be completed by December 2008				
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INTRODUCTION

Preventive strategies require identification of cancer-susceptible individuals resulting from combinations of carcinogen exposure and lack of protective factors. Oxidative reactions have been implicated as important modulators of human health and can play a role in both disease prevention and disease development. A large number of studies have demonstrated an increased oxidant burden and consequently increased markers of oxidative stress in the airspaces, breath, blood, and urine of smokers and of patients with chronic obstructive pulmonary disease (COPD) [1,2]. Changes in dietary habits with the intake of more cancer-chemopreventive agents appear to be a practical approach for cancer prevention in subjects with increased oxidative stress as is the case of subjects with COPD and ≥ 25 pack/year of smoking history.

The present study will investigate the ability of regular green and /or black tea consumption to decrease oxidative stress during the context of a randomized, controlled, double blinded, dietary intervention trial. Levels of 8-hydroxydeoxyguanosine (8-OHdG) will be used to measure DNA damage and levels of 8-F2 isoprostanes (8-epi-PGF2) and ethanes will be used to measure lipid damage. Testing for biomarkers of oxidative stress in exhaled breath condensate (EBC) will complement other innovative methods currently being investigated. The use of this novel strategy might enable further classification of people at risk of increased oxidative stress lung cancer, such as smokers, workers in nuclear weapons plants, Gulf War veterans [3], and US Marines by degree of risk. Such refinement of risk analysis might then be used to identify candidates for screening studies.

BODY

Task 1. Preparation, protocol development and analysis of tea extracts and placebo (QC/QA) for tea polyphenols (Months 1-7)

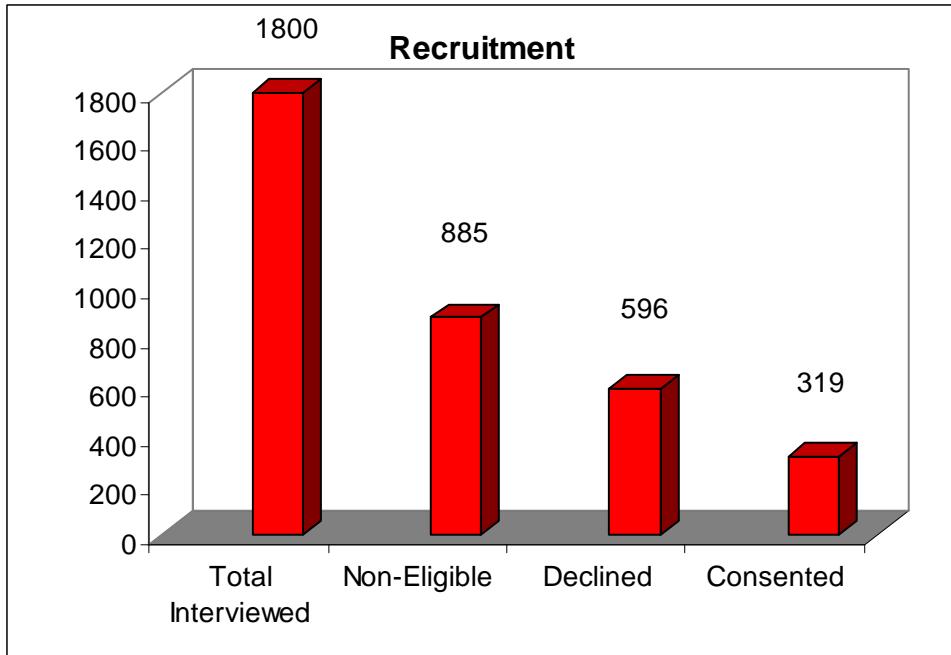
Completed: See previous reports

Task 2. Recruitment/ eligibility, Run-In & baseline assessment of oxidative stress (Completed)

Recruitment of eligible subjects started in month 5 of the study and was completed in December 2007. During the last 3 years of recruitment, we found that we have to screen more than 1500 subjects to be able to find 300 eligible subjects and 40 % of the subjects are more likely to drop-out during run-in (before randomization) due to various reasons . Therefore, we expended the recruitment time to be able to have 150 subjects completing the study.

A total of 1800 subjects were interviewed by phone for eligibility criteria. Subjects were not eligible because of age, pack/year of cigarettes, medications, had cancer, or currently enrolled in another study. The main reasons for refusing to participate in the study were not willing to give up tea, cannot drink much tea, or the long duration of the study.

Recruitment was successfully completed by the end of July 2007 and subjects were enrolled and followed up for the 6-month intervention period. All eligible subjects completed all baseline questionnaires and started the run-in period. Each enrolled participant, received 1-month of placebo tea bags, study teacup, a 3-minute timer, the monthly diary and health monitoring forms, and sterile urine cups. Subjects were contacted biweekly to ensure and encourage adherence and to monitor any adverse event. Subjects who completed the run-in period provided blood, urine and exhaled breath condensate (EBC) samples for biomarker analysis. All subjects (100%) provided buccal cells and 65% of the subjects provided induced sputum samples for storage.

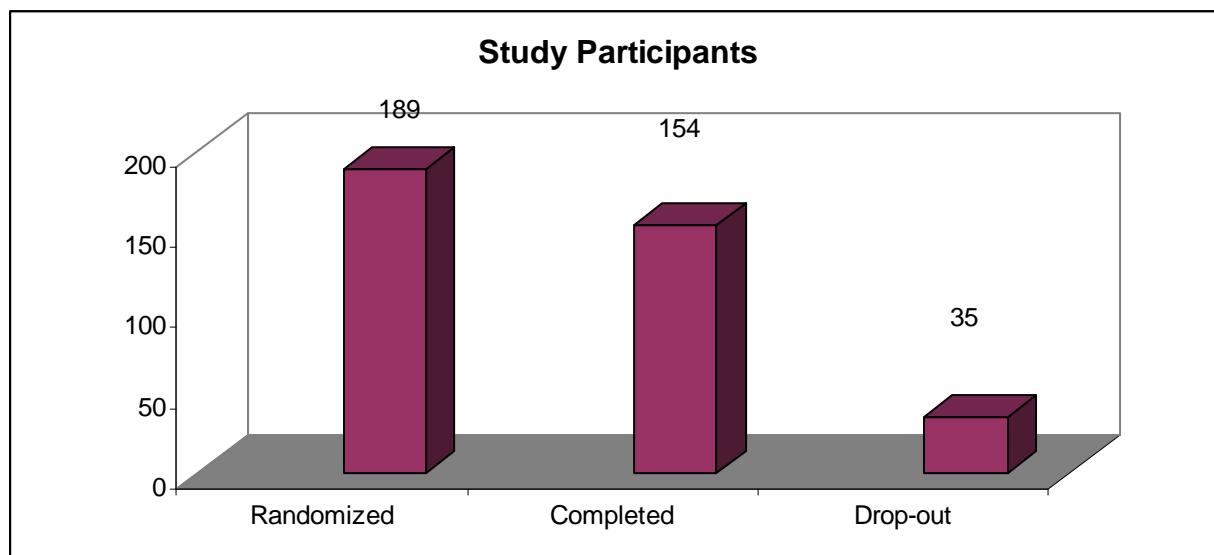


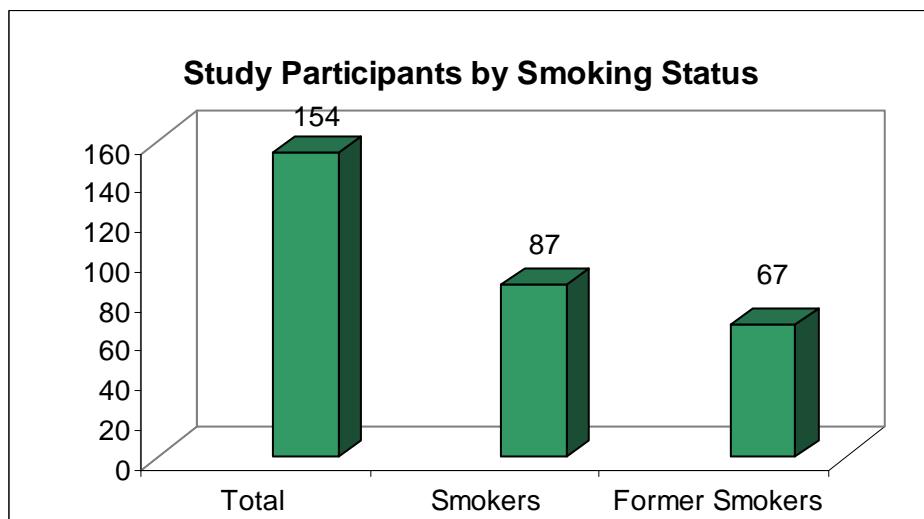
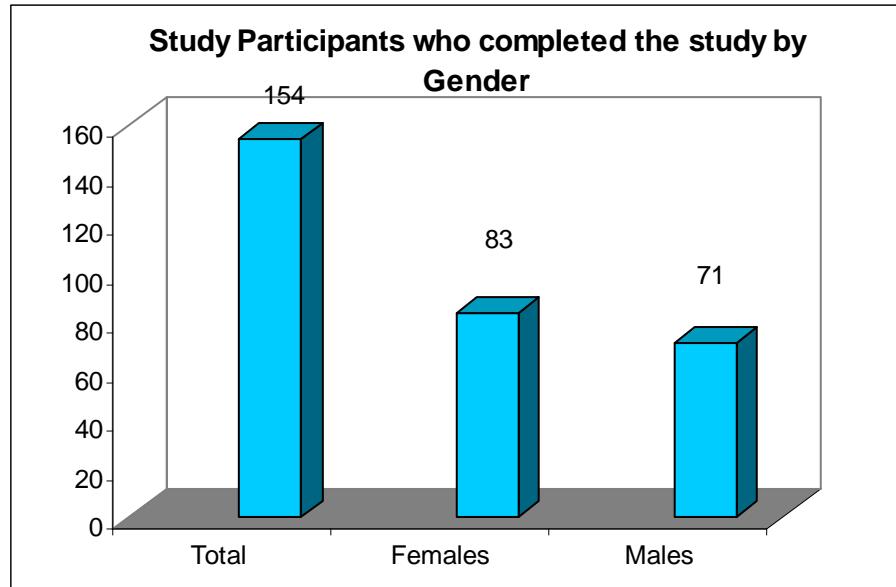
By the end of December (2007), 319 participants signed the consent form and were screened for confirmation of COPD eligibility criteria (spirometry for lung function tests)

Task 3. Intervention, Follow-up & Exit focus groups to study the effect of tea consumption on DNA (8-OHdG) and lipid (8-epi-PGF2) damage in blood, urine, and EBC (Completed).

a) Randomize eligible COPD chronic and former smokers into one of three interventions: black tea, green tea or placebo for 6 months.

A total of 154 subjects have completed the study and they have been randomized to 1 of the 3 arms of the study. The demographics of the study population (subjects who completed the study) are shown below.





b) To maintain high adherence to the study intervention including collection of blood, urinary, and EBC samples through the 6-month intervention period and 1-month follow-up period.

Study participants were contacted biweekly by phone to ensure adherence. Subjects completed a tea and smoking diary in which they reported their daily intake of tea (amount and time) and the number of cigarettes smoked each day. They also completed a health monitoring form in which they report any change in medication use, any health-related event, or any perceived adverse event. Data is being entered for the last cohort.

c) To identify issues affecting recruitment and retention of chronic and former smokers with COPD in a lung cancer prevention trial.
 d) To determine whether subjects will continue to consume tea regularly after the end of the intervention.

Exit and satisfaction questionnaire were collected from all participants that completed the study. Data is being entered into the computer database. This is an ongoing process and final results will be available at the end of the study when all data will be entered and analyzed. To date, the most common causes of drop-out are too much fluid and time commitment.

Task 4. Laboratory analyses and data entry (Ongoing)

a) Quality control assurances of laboratory methods

We have completed all the validation and quality control measures for the biomarkers of oxidative stress. Our quality control and validation data show that the urinary biomarkers of oxidative DNA and lipid damage are stable even when left at room temperature for 3 consecutive days.

b) & c) Urine & Blood Oxidative Stress biomarkers' analyses and quality control

Laboratory analyses of urinary and blood biomarkers of oxidative damage started on time as scheduled. All laboratory analyses undergo quality control/quality assurance measures before being sent for data entry. This preliminary summary represents the data that had been entered into our database. This is an ongoing process. All laboratory analyses will be completed by end of June 2008.

Measurements of 8-hydroxy-2'-deoxyguanosine (8OHdG) in human urine and lymphocyte DNA by high performance liquid chromatography-electrospray tandem mass spectrometry

A method for quantification of 8OHdG in human urine by HPLC-tandem mass spectrometry has been implemented and validated in Dr. Chow's laboratory. The analysis is performed on a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometric system in tandem with a Surveyor LC system. The urine sample (50 µl) is diluted 1:1 with water and injected onto the HPLC system. HPLC separation is achieved with a BDS Hypersil C₁₈ column (150 x 2.1 mm, 5µ) and a gradient mobile phase. The gradient starts at 1% methanol and 99% 10 mM ammonium formate and is increased linearly to 80% methanol and 20% ammonium formate by 15 minutes. The system is re-equilibrated with 1% methanol and 99% ammonium formate for 5 minutes before the next injection. The flow rate is 0.2 ml/min. 8OHdG (from precursor ion *m/z* 284 to product ion *m/z* 168) and 2'-deoxyguanosine (from precursor ion *m/z* 268 to product ion *m/z* 152) are detected with multiple reaction monitoring (MRM) in the positive ion mode utilizing electrospray ionization. Linear calibration curves have been established from 0.3 to 30 ng/ml (1-100 nM). The within-day and between-day coefficient of variation of the assay is less than 10%. 8OHdG is found to be stable in urine when stored at room temperature for 72 hours.

Dr. Chow's laboratory has also tested various DNA digestion procedures for measurement of 8OHdG levels in DNA to maximize release of normal nucleosides and 8OHdG and minimize oxidation of 2'-deoxyguanosine and DNA during sample preparation and handling. Dr. Chow's lab had optimized the procedures for isolating DNA from blood lymphocytes for 8OHdG measurements. All laboratory analyses are ongoing. Representative LC/MS/MS chromatograms for 8OHdG (*m/z* 284/168) have been presented before (See previous report).

Measurements of 8-isoprostaglandin F_{2α} (8-iso-PGF_{2α}) in human urine by high performance liquid chromatography-electrospray tandem mass spectrometry

A method for quantification of 8-isoprostaglandin F_{2α} in human urine by HPLC-tandem mass spectrometry has been implemented and validated in Dr. Chow's laboratory. The analysis is performed on a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometric system in tandem with a Surveyor LC system. The urine sample (1 ml) is extracted with a solid phase extraction procedure before injecting onto the HPLC system. Isotope labeled 8-isoprostaglandin F_{2α} -D4 (8-iso-PGF_{2α}-D4) is used as the internal standard. HPLC separation is achieved with a BDS Hypersil C₁₈ column (150 x 2.1 mm, 5µ) and a gradient mobile phase consisting of 2 mM ammonium acetate (A) and 5:95 methanol:acetonitrile (B). The gradient starts at 20% B and increases linearly to 35% B by 27 minutes. The system is re-equilibrated with 20% B for

10 minutes prior to the next injection. Flow rate is 0.2 ml/min. 8-iso-PGF_{2 α} (from precursor ion *m/z* 353 to product ion *m/z* 193), 8-iso-PGF_{2 α} -D4 (from precursor ion *m/z* 357 to product ion *m/z* 197), and prostaglandin F_{2 α} (from precursor ion *m/z* 357 to product ion *m/z* 197) are detected with multiple reaction monitoring (MRM) in the positive ion mode utilizing electrospray ionization. Linear calibration curves have been established from 20 to 5000 pg/ml. The within-day and between-day coefficient of variation of the assay is less than 10%. Representative chromatograms for (8-*iso*-PGF_{2 α}) have been presented before (See previous report).

We have completed the urinary analyses of biomarkers of oxidative DNA damage (8-OHdG) and lipid damage (8-F2 isoprostanes), and creatinine for the first and second group of subjects who completed the 6-month study. Biomarkers were measured at baseline, month 3 (mid-intervention), and month 6 (end of intervention). Because of the nature of the study (randomized and blinded), we will not be able to sort the data by randomization group until the end of the study, and hence, we will not be able to comment on the effect of tea drinking until the end of the study. Laboratory analyses is ongoing for the last group of subjects who completed the study in December 2007. Summary of the overall entered data is presented in the Table below.

d) Oxidative stress biomarkers in exhaled breath condensate

Measurements of 8-isoprostaglandin F_{2 α} (8-*iso*-PGF_{2 α}) in human exhaled breath condensate

A commercially available enzyme immuno assay kit (Cayman Chemical, Catalog No. 516351) which has shown a limit of quantification of 4 pg/ml for measurements of 8-*iso*-PGF_{2 α} levels in the breath condensate samples. We have established a reproducible 8-*iso*-PGF_{2 α} calibration curve from 3.91 to 500 pg/ml. The between-day and within-day coefficient of variance is less than 11%. A number of baseline exhaled breath condensate samples were tested using this procedure and the 8-*iso*-PGF_{2 α} levels were found to be at the low end of the calibration curve (2-4 pg/ml). With a concentrating factor of 10, we were able to observe absorbance readings comparable to those observed with concentrations of 20-30 pg/ml. Because this is a more reliable concentration range to monitor any modulating effects from tea intervention, we plan to concentrate all exhaled breath condensate samples by a factor of 10 prior to sample analysis. Exhaled breath condensate samples are currently being analyzed.

Measurements of Nitric Oxide (NO) and Ethane in Exhaled Air

Measurements of NO and ethane in exhaled air is being done at baseline and month 6 (end of intervention). All laboratory analyses undergo quality control/quality assurance measures before being sent for data entry. This preliminary summary represents the data that had been entered into our database. This is an ongoing process. Summary of the overall entered data is presented in the Table below.

<u>Visit</u>	<u>Nitric Oxide (ppb)</u>	<u>Carbon Monoxide (ppm)</u>	<u>Ethane (ppb)</u>
	Range	Range	Range
Baseline	5.2 – 77.0	0 – 52.0	0.4 – 14.8
Month 6	8.1 – 85.0	0.3 – 32	0.5 – 21.4

Antioxidant levels in blood

We have completed the analyses of blood antioxidants for the first 2 groups of participants who completed the study. Data entry and laboratory analyses for the third cohort is ongoing

Antioxidant Enzymes Data

<u>DOD</u>	CAT (nmol/min/g Hb)	GPx (nmol/min/g Hb)	SOD (U/g Hb)
Mean	597,248.98	26,585.17	5,366.94
Standard Error	10,804.46	549.13	108.93
Median	578,872.52	24,729.50	5,041.89
Standard Deviation	174,551.37	9,286.64	1,842.10
Sample Variance	30,468,179,876.48	86,241,725.00	3,393,341.62
Range	1,258,631.88	57,483.55	10,365.73
Minimum	209,148.44	9,711.17	1,643.06
Maximum	1,467,780.32	67,194.73	12,008.79
Sum	155,881,983.83	7,603,357.63	1,534,944.93
Count	261.00	286.00	286.00
Confidence Level(95.0%)	21,275.37	1,080.87	214.40

Development of the methodology for RNA extraction from sputum

We have successfully developed the methodology for RNA extraction from sputum with a significant yield of RNA. Preliminary testing of gene expression of proliferation and apoptosis are successful. Stored sputum samples will be analyzed during the coming 6 months and data will be reported in the final report.

KEY RESEARCH ACCOMPLISHMENTS

- Development and approval of the study protocol
- Development and approval of all study forms and questionnaires
- Successful recruitment and screening
- Successful enrollment in the study
- Successful collection of biological samples (blood, urine, EBC, buccal and sputum samples)
- Validation and quality control of all laboratory methods
- Ongoing laboratory analyses of biological samples.
- One hundred and fifty four participants successfully completed the study.
- Successful development of methodology for RNA extraction from sputum
- Successful measurements and analyses of RNA gene expression in sputum samples

REPORTABLE OUTCOMES

None for year 2007: the focus was on completing the study on time. At least one manuscript will be ready by the end of this last year.

CONCLUSIONS

During the last 5 years of the study, we were able to reach a large number of potential participants. We interviewed (initial screening) 1800 subjects and randomized 189 eligible subjects in the study. Identification of eligible participants was a challenge, however, we were successful in reaching a large pool of potential subjects. We have accomplished our goal and sample size by having 154 subjects completing the study. Laboratory analysis is ongoing and should be completed by end of June 2008. The data analyses, final report and manuscript will be completed by end of December 2008.

Because tea is one of the most popular beverages consumed worldwide, the relationship between tea consumption and human cancer incidence is an important concern. Tea can be easily consumed with one's ordinary meals making compliance and adherence to dietary intervention more likely to succeed. Thus, the role of tea drinking as a potential inhibitor of carcinogenesis merits careful evaluation. We believe that a

program of nutritional intervention with realistic dietary modifications that are effective, safe, and acceptable should be the cornerstone of any cancer prevention strategy.

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